

The Role of F-actin in Hyphal Branching

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Abstract

Hyphal organisms are a commonly used model system for studies of polarised growth. While growing hyphal tips offer a good example of polarised growth, little detail of the process of polarisation can be determined from them. Hyphal branching offers a good example of the development of polarity, however to date it has been largely impractical to study hyphal branching, due to the irregular timing and location along the hypha of natural branch formation. Chemical induction of branches circumnavigates this problem, using a localised concentration of nutrients adjacent to the growing hypha to stimulate controlled branching.

Using previous studies of hyphal branching combined with the current understanding of hyphal tip growth, a model of the branching process was established (Jackson *et al.* 2001). Reception of a branching cue leads to the formation of a radial F-actin array at the new branch site. This, by means of either delivery of cell wall softening enzymes or direct mechanical pressure, leads in turn to the emergence of a visible bump in the hyphal wall. This bump enlarges and then progresses into the branch proper. The bump stage of the branching process is perhaps the least understood, with existing studies giving detail of pre- and post-bump events. The research described in this thesis suggests that bump emergence is a two stage process; an early bump stage, where localised cell wall softening leads to turgor pressure in the cell pushing out the bump, and a late bump, where F-actin is arranged into the developing branch. The addition of an F-actin inhibitor to the induction solution confirmed that the early bump stage is relatively independent of

the F-actin cytoskeleton, however this experiment was unable to test F-actin's role in full branch development.

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Abbreviations

ABP	Actin Binding Protein
ADF	Actin Depolymerising Factor
DMA	Defined Media (low in amino acids)
DIC	Differential Image Contrast (Microscopy)
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
F-actin	Filamentous actin
G-actin	Globular actin
Lat B	Latrunculin B
LMP	Low Melting Point (agar)
μm	Micrometre
μM	Micromoles
min	Minutes
Min. salts	Mineral Salts (solution)
M	Molar (moles per litre)
%	Percent
PIPES	Piperazine-N,N'-bis-[2-ethanesulfonic acid]
pH	Potential of Hydrogen
PYG	Peptone, Yeast and Glucose media
s	Seconds
T	Temperature

t	Time
UV	Ultra-violet

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Chapter 1

Introduction

Cellular polarisation is a key factor for growth in all complex, eukaryotic cells. For cellular polarisation to occur there must be a framework for the organelles to be organised against, this framework is formed by the cytoskeleton, specifically the actin microfilaments (F-actin) and microtubules. Fungi and oomycetes, which are hyphal organisms, have long been used as model systems for polarised growth studies, as their shape and mode of growth at the hyphal tip are obvious examples of this type of growth. This is also the case for other tip growing systems such as pollen tubes, root hairs and algal rhizoids. While growth at the tip is well studied, the process of hyphal branching in fungi and oomycetes is less well studied, due to the unpredictable timing of branch formation. F-actin has been suggested to play a central role in tip growth in hyphae, and is hypothesised to play an equally important role in the process of branch formation. With the development of techniques that can induce the formation of a branch in hyphae, the early stages of branch formation and the role of actin in this process can be studied.

Cell polarity

Cell polarity is central to cellular growth. Polarity allows directional growth and allows specialisation of distinct areas of cells. Polarised domains have previously been studied in algal zygotes (Kropf, 1997), pollen tubes (Callaham and Hepler, 1991) and oomycete

hyphae (Jackson and Heath, 1993). Fungal and oomycete hyphae have been used as models of cellular polarisation due to their distinctly localised mode of growth.

There are two major factors identified in the process of hyphal tip growth, cellular turgor pressure and the actin cytoskeleton. Traditionally, turgor pressure linked to a softening of the cell wall at the growing tip was believed to be responsible for hyphal apical extension (Bartnicki-Garcia, 1972). In such models of growth the actin cytoskeleton plays a key ancillary role, delivering the vesicles containing cell wall material and cell wall softening enzymes to the tip of the hyphae. A softened wall (or one in which substantial cross-linking of wall polymers has not yet occurred) at the tip was thought more likely to yield to the force of turgor than the unsoftened wall in subapical regions (Wessels, 1986). This led to localised cell expansion at the tip, and hence the process of tip growth. However, the discovery that oomycete hyphae could continue growing with low internal turgor pressure necessitated revisions of such models. The amoeboid growth model was established, where the F-actin cytoskeleton mechanically pushes the growing tip forward.

In oomycetes, a distinct F-actin cap is apparent in the growing hyphal tip (Heath, 1987). Under the turgor driven model, it was hypothesised that this was involved in vesicle delivery to the growing tip and also that the microfilaments themselves could along with the cell wall resist turgor and thus play a role in regulating tip yielding. However, the amoeboid growth model implies a much more active role for the apical F-actin cap in tip extension. In this model, hyphal tip growth is likened to the movement of tube-dwelling amoebae. Heath (1995) proposed that the F-actin cytoskeleton was linked to the

plasmalemma and the cell wall possibly by integrin like proteins (Chitcholtan and Garrill, 2005). These allow the cell to adhere to the substrate, the bulk of the cytoplasm moves forward akin to the movement of an amoeboid pseudopodia, then the cell wall components are exocytosed for assembly of the new wall (Harold *et al.*, 1996).

Finally, it has more recently been suggested that both modes of growth could occur concurrently in the same mycelium, which one depending on the hyphae's surrounding conditions. Certainly tip growth is likely to involve a dynamic F-actin cytoskeleton as invasive growth (i.e. growth through agarose media) of the oomycete *Achlya bisexualis* has been shown to involve the formation of an F-actin deplete zone at the hyphal tip and significant weakening of the hyphal cell wall. In contrast non-invasive growth (i.e. growth on top of agarose growth media or through liquid media) yields no F-actin deplete zone and a stronger wall (Walker 2004). These observations also appear to be true for other oomycetes such as *Phytophthora cinnamomi* and also for the fungus *Neurospora crassa* (A. Garrill, personal communication).

While hyphal tips are useful for the study of established polarised zones and the maintenance of polarity, they are of less use in studies of the development of polarisation. The process of hyphal branching presents a system on which studies of the initial generation of polarity is possible. In view of this Morris (1999) proposed that the induction of hyphal branches presents a means of studying the development of polarised regions in cellular growth.

Branching and Branch Induction

As detailed above, filamentous fungi and oomycetes grow at the tips of their hyphae. This process alone would at any one time yield one-dimensional growth and limit the cell surface area and therefore nutrient absorption. To spread and increase their surface area hyphae branch, creating new growing tips that are able to spread between existing hyphae, ultimately forming what can be very dense mats of hyphae called mycelia.

Fungi have been shown to be very efficient in their nutrient absorption, balancing their total length of hyphae with their area of active nutrient absorption. To achieve this balance they are believed to alternate between the so-called Phalanx and Gorilla growth strategies. When faced with a new environment, a hyphal organism will revert to a structured “phalanx” mode of growth, with regular, ordered branching. In this mode of growth the average number of branches per length of hyphae remains constant in the same media at the same temperature (Trinci *et al.*, 1994), however the location and timing of branch formation is largely random. When an area of high nutrient concentration is encountered by a growing hypha, it switches to a localised aggressive “gorilla” phase of branching in order to rapidly maximise the hyphal area and exploit the new site of nutrients. These dual modes of growth make predicting natural branch emergence impossible, due to the random timing and location of branches along hyphae, but ironically they also give a method of artificially inducing branches, by creating localised high nutrient areas along a growing hypha.

Originally, experiments on branching involved whole mycelia, the branching frequency of individual hyphae where recorded when mycelia were presented with an abundance of a nutrient or an inhibitor of a key pathway. These experiments firstly showed that branching increased in a high nutrient environment, but that this came at the expense of decreased tip growth (Trinci *et al.*, 1994). The ability to increase branching on entire mycelia led to the subsequent development of a technique for localised branch induction. This was possible through the localised application of nutrients next to the hyphae via a micropipette (Schreurs *et al.*, 1989, Morris, 1999) (Fig. 1.1). This technique has been shown able to induce a branch in 85% of attempts. It should be noted that branches can form by two distinct mechanisms. Apical bifurcation is the process where a hyphal tip divides down the centre to form two growing tips. Alternatively, hyphae can branch subapically; new growing tips emerging at 90° to the long axis of the original hyphae (Trinci, 1971). It is in this latter form of branching that is of interest in the study of Morris (1999) and in the current study.

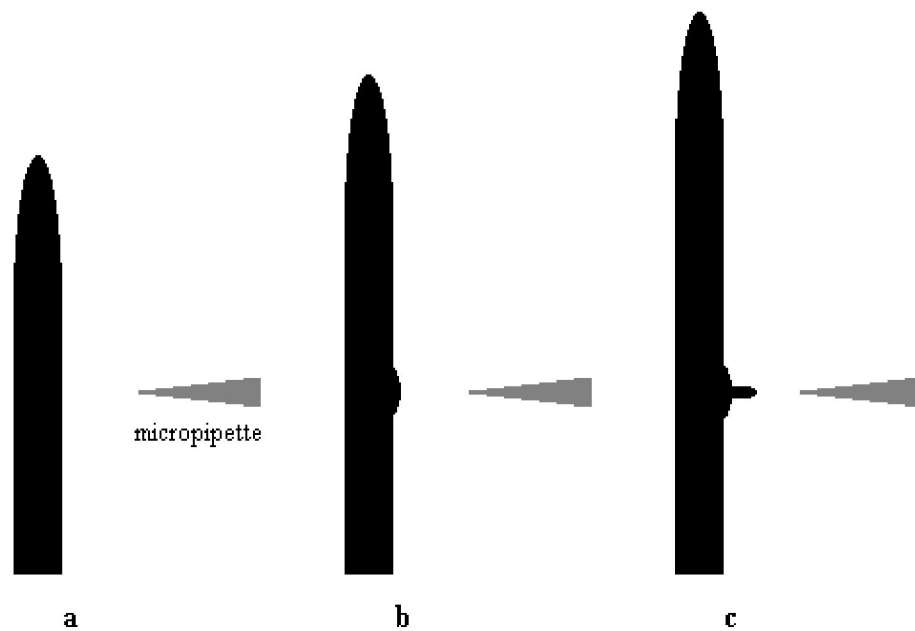


Figure 1.1. Diagrammatic representation of localised branch induction of *Achlya*. Hyphae of *Achlya* were induced to branch by placing a phenylalanine-filled micropipette adjacent to a growing hypha (a). The first sign of branch formation is the appearance of wide bump in the hypha near the pipette. This occurs approximately 20 minutes after placement of the micropipette (b). Shortly after the appearance of the bump a branch emerges (c). Initially the branch is thin but will gradually increase in girth. (From Jackson et.al. 2001)

Other chemical methods of branch induction exist, with varying conditions and results. Application of the steroid hormone antheridiol to hyphae of *Achlya ambisexualis* has been shown to lead to increased branching (Mullins, 1979). However, rather than giving normal vegetative hyphae, this technique results in the formation of a reproductive primordial while also causing the cessation of tip growth. While localised induction with these hormones is theoretically possible, to date it has not been attempted, with studies limited to their addition to whole mycelia (Gow *et al.*, 1987). These complications limit the applications of this method to studies of the reproductive structures of hyphae.

In addition to the chemical technique just described, there are luminescent means of branch induction. Very short wavelength light, in the blue-UV region of the electromagnetic spectrum (i.e. the 360-430nm region), has been used to induce branches in *Gigaspora gigantea* (Chen *et al.*, 2002). Branching could be induced on any site on the hyphae, aside from the apical most 2mm. However, the technique has only been applied to a select few groups of fungi, and the resulting branch response was uncontrollable, multiple branches frequently occurring from one induction site, and often around the induction site as well. UV microbeams are also very damaging to growing hyphae, often leading to cell rupture when high doses are used. Continuing research into luminescent induction of branching is focussing on the genetics behind the response (Lauter *et al.*, 1998). With respect to the current study UV microbeams have also been shown to severely affect the arrangement of the F-actin cytoskeleton (Jackson and Heath 1993). Thus it is not possible to use them as it would not be known if any observed

change in F-actin reflected a role that it was playing in the branching process or if the change was due to UV-invoked damage.

While the above techniques each potentially allows for a branch to induced to some degree of accuracy only the chemical method of induction allows for the most accurate prediction of exactly where the branch will occur, therefore it was the technique that was used in this thesis.

Branching Model

A model of the process of hyphal branching was proposed by Jackson, Morris and Garrill (2001). In this model the first stage of the branching process was proposed to be the reception and transduction of a directional cue. This can take the form of an external signal or be intrinsically controlled by the hypha itself. The cells reaction to the cue is the formation of a polarised domain; this is where the future branch will form. This localised polarisation leads to the transport of vesicles containing cell wall softening lytic enzymes to the branch site, leading to the formation of a bump in the hyphal wall, which in turn leads to the emergence of the branch proper. This process is depicted in Figure 1.2.

The initial cue for branch induction remains unknown. Calcium ions have been tested but do not appear to be the cue (Morris, 1999); the conclusion of Morris' study was that pH was the most likely signal, although this possibility has not yet been directly tested. Intrinsic control of branching, the other possible cue for branch initiation, has been

suggested in view of the subapical accumulation of materials such as vesicles that are needed for tip growth (Carlisle and Watkinson, 1994, Trinci *et al.*, 1994).

The reception of the branching cue leads to the development of a polarised domain. Traditionally, bud emergence in yeast has been used as a model system of polarised domain formation (reviewed by Harris and Momany, 2003). In yeasts the bud site is determined by the presence of cortical markers working with a Ras-related GTPase module. The positional information is transduced through the Cdc42 GTPase to the F-actin cytoskeleton and secretory apparatus, which are arranged by means of the formins and septins for the process of actual bud formation. While it is believed that a similar process may occur in hyphal fungi, the role of the cortical markers may be performed by activated receptors, as occurs in hyphal mating, or may indeed be discarded altogether with branch emergence being completely random. Irrespective of this, the signalling pathways that lead to branch formation fall outside of the scope of this research, which focuses on the mechanical process of branching. Thus these will not be considered further but could form the basis of future studies.

For the purpose of the model proposed by Jackson *et al* (2001), the polarised domain is believed to take the form of an accumulation of F-actin in the hyphae, with microfilaments radiating from the branch site. This network could serve two possible functions, which are suggested in the model above. The first of these is enabling the delivery of vesicles containing cell wall softening lytic enzymes and other branch formation materials, alternately this network could be used by motor proteins to exert

mechanical pressure on the branch site, physically pushing the branch out from the hyphae (Brachewich and Heath, 1998). The softening of the cell wall at the branch site coupled with turgor pressure and/or protrusive force generation by the F-actin cytoskeleton leads to the formation of a visible bump in the hyphae. As the branch proper is formed the mechanisms of tip growth in a hypha, including an F-actin cap, are assembled, ready for the new branches continued growth. This model places significant emphasis on the role of the F-actin cytoskeleton in branch formation. It is this that this thesis aims to investigate.

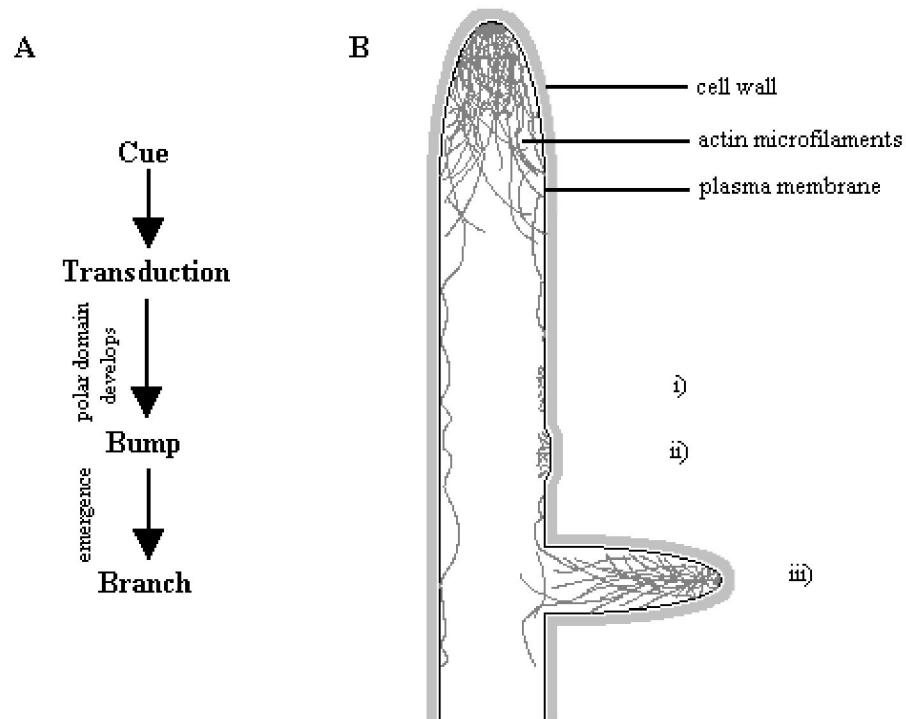


Figure 1.2. A) Flow diagram indicating the sequential progression of branch formation. B) Illustration of the changes that take place along a hypha as a branch forms; I) accumulation of actin at the cortex, ii) cell wall softening by lytic enzymes and bump formation, iii) the branch grows out. (From Jackson *et al.*, 2001)

Actin

The cytoskeleton is a ubiquitous feature of eukaryotic cells. It consists of microfilaments, microtubules and intermediate filaments, all composed of proteins, each with unique roles in cellular functioning. The cytoskeleton provides the framework necessary for cellular polarisation, growth, movement and reproduction. Microfilaments and intermediate filaments both give structural support to the cell, while the microfilaments also play a role in cytoplasmic streaming, vesicle transport and cellular movement (Alberts *et al*, 2002). Microtubules are involved in the movement of membrane bound organelles throughout the cell and intracellular signalling. Despite their differing roles, these structural components are all functionally interlinked by cellular machinery and shared accessory proteins.

Actin exists in two main forms. G-actin is the monomeric form that exists freely in all eukaryotic cells. Polymerisation of this G-actin leads to the formation of a linear actin strand. This second form of actin, microfilaments or F-actin, consists of two of these linear polymers interweaved helically. It is these actin microfilaments that are believed to be able to provide major mechanical force and structural support necessary for tip growth, and are hypothesised to play a similarly central role in branching.

The actin cytoskeleton is a highly dynamic structure in a constant state of reorganisation as the cell grows. As the actin microfilaments are central to growth and are thus frequently rearranged, a complex series of actin binding proteins (ABPs) have evolved

facilitate this process. Cofilin/ADF (actin depolymerising factor) is a key element of this rearrangement of the microfilaments. This enzyme controls the severing and depolymerising of microfilaments, in the process freeing actin monomers for new construction. Since new branch sites are believed to involve significant rearrangement of the actin cytoskeleton *in situ*, it is possible that increased ADF activity is present around the new branch site. Other ABPs found in hyphal organisms include profilin, controlling microfilament formation, capping protein, which regulates filament length, and fimbrin/SAC6, which facilitates microfilament bundling.

In oomycete hyphae the actin cytoskeleton that is observed in cells can be divided into three major structural components, apical F-actin caps, peripheral F-actin and peripheral F-actin patches, also known as F-actin plaques. Apical F-actin caps are believed to play a major role in tip growth, whether turgor- or amoeboid-driven. In the oomycete *Saprolegnia ferax*, apical F-actin caps have been shown to be highly dynamic in structure but largely consistent between hyphae, with a dense mesh of filamentous actin at the hyphal tip, which gradually coarsens as it spreads from the tip, until it merges into the peripheral F-actin cytoskeleton in the main body of the hyphae (Heath, 1987). These F-actin caps were also observed in the same study in natural, non-induced, emerging branches in growing hyphae.

The peripheral F-actin microfilaments in the main, sub-apical body of the hyphae are thought to serve a number of roles. F-actin has been implicated in organelle movement and positioning, vesicle transport, cytoplasmic migration and localisation of plasma

membrane associated proteins (Bachewich and Heath, 1997). In hyphal cell division the F-actin cytoskeleton forms a contractile ring around the plane of division, assisting in the process of septation.

The purpose of the F-actin plaques is uncertain, they are believed to play a role in endocytosis (Engqvist-Goldstein & Drubin, 2003) and supporting cell wall disposition (Utsugi *et al.*, 2002). Actin plaques typically consist of 85 20-actin subunit filaments associated with the ARP2/3 complex, which facilitates plaque formation, and the ABP fimbrin/sac6, which contributes to plaque stability (Young *et al.*, 2004). They are also readily visualised when stained, thus greatly aiding the observation of the F-actin cytoskeleton.

As branching is a mechanical process the cytoskeleton is heavily involved. Microtubules and intermediate filaments have both been shown to play ancillary roles in hyphal growth; normal growth, although slowed, is possible in the presence of inhibitors. However, growth has been shown to cease in the presence of actin inhibitors, indicating that actin is the main structural element involved in hyphal growth (Heath *et al.*, 2000). As previously mentioned, the role of F-actin in tip growth in hyphae has been well studied (Walker, 2004), but the aforementioned difficulties in studying hyphal branch formation have prevented any localised direct study of the branch process, previous experimentation on the subject being limited to whole plate manipulations of cultures (Heath *et al.*, 2000).

Latrunculin

Latrunculin B is a marine toxin isolated from the Red Sea sponge *Latrunculia magnifica*. It acts by inhibiting actin polymerisation in cells it comes into contact with, thereby disrupting all actin microfilament processes in the effected area. It has been widely used in studies of the actin cytoskeleton (Gupta and Heath (1997), Brachwich and Heath (1998)), proving significantly more effective than the previously used cytochalasins. Thus the addition of latrunculin in the proposed experiments would allow investigation of the relative importance of F-actin in the process of hyphal branching.

Latrunculin has been used frequently in previous studies of the actin cytoskeleton, but again only in whole plate applications. The disruption of the actin cytoskeleton has been shown to lead to ballooning of the exposed cell, confirming F-actin's role in delivery of growth vesicles (Heath *et al.*, 2003). More recently it has been shown that the action of latrunculin disrupts the radial actin arrays that precede hyphal branching, leading to the complete disruption of branching (Varvarigos *et al*, 2004). This study adds additional credence to the proposed centrality of F-actin's role in the branching process suggested in the model, however it was performed in protoplasts obtained from the alga *Macrocystis pyrifera* gametophytes, therefore it's applicability to the oomycete system used in this experiment is uncertain.

Fixatives

Traditionally in studies of the cytoskeleton hyphae have been fixed with formaldehyde solution before staining. Formaldehyde has many advantages; most significantly it's lack of fluorescence under the confocal and epifluorescent microscope, which effectively improves the ability to detect the subsequently stained cytoskeleton. It's drawbacks, however, are that the fixation given is not permanent, thus while the cells are being stained and observed finer detail may be lost due to reversal of the fixation. Superior fixatives, such as glutaraldehyde, give a more permanent fixation, due to their multiple binding sites, but this comes at the expense of high background fluorescence. An additional dialdehyde has recently been found to give superior fixation without the high background fluorescence of glutaraldehyde. Thus a combination of methylgloxal and formaldehyde has been found to give markedly better fixation than formaldehyde alone, enabling detail to be captured in imaging that previously wasn't possible. This has led to the description of a new structural component of the F-actin cytoskeleton in oomycetes, an F-actin deplete zone (Yu *et al.*, 2004). As described earlier this appears to be present predominantly in hyphae that are growing invasively (i.e. through agarose media) compared to those that are growing non-invasively (i.e. on top of agarose media or through liquid media) (Walker 2004). This deplete zone may allow localisation of turgor forces or alternatively localisation of vesicle deposition. Irrespective of the role of the F-actin deplete zone the combination of chemical induction of branches and better

preservation of the F-actin cytoskeleton together make the in depth study of the processes of hyphal branching possible.

Aim of the Thesis

This thesis investigates the role of the F-actin cytoskeleton in the process of hyphal branching in the oomycete *Achlya bisexualis*. The Oomycetes have proven useful model organisms for the study of tip growth due to their large hyphal sizes. The role of F-actin was studied by firstly inducing a series of bumps and branches of varying lengths then staining the cells for F-actin and observing changes as the branches form. In a second series of experiments, inductions were performed with the addition of 2.5 μ M latrunculin-B, an actin inhibitor, to the inducing media. It was hypothesised that actin played a key role in branch formation, and that treatment with latrunculin-B would lead to the cessation of branch formation. The results of this thesis suggest that while bump formation may not require the major reconstruction of the localised actin cytoskeleton, for the bump to progress to a full branch actin is essential.

Chapter 2

Materials, Methods and the Development of the Induction Assay

Organism

Achlya bisexualis, a female strain originally isolated at the University of Canterbury from *Xenopus laevis* dung and maintained as part of the University of Canterbury culture collection, was the organism used in these experiments. This was chosen on the basis of the large hyphal size, facilitating micromanipulation, ease of fixation and staining and in view of previous successes with the species in branch induction trials (Morris 1999). Stock plates were kept incubated in the dark at 20°C on PYG media, a high nutrient non-defined media (details of this media are given in Appendix 1).

Spores

In a previously reported study of branch induction, (Morris 1999), hyphae of *Achlya bisexualis* were isolated from a growing colony. Basically *A. bisexualis* hyphae were grown from agar plugs that had been placed on cellophane lined DMA agar plates. The brand of cellophane used was Hallmark brand, which had been boiled three times to remove plasticisers (Robson *et al.*, 1991). A 1cm square piece was cut from the edge of the growing margin of the colony and transferred to a well plate slide, the hyphae were washed off the cellophane then covered with 2% low melting point DMA agar, which in turn was covered with DMA broth. This procedure worked well for branch initiation

studies, as the hyphal mat provided an abundance of growing hyphal tips for study; once one hyphae had been induced, the next hypha along the mat could be used. However, if the slide is moved from one microscope to another, as was necessary in this study, the use of hyphal mats becomes problematic. The mass of morphologically very similar hyphae that are growing in close proximity and parallel to one another means that induction sites are likely to be difficult to identify after the transit from one microscope to another during fixing and staining. This is especially relevant in studies where the early stages of branching are of interest.

The proposed solution to this problem was to use germinating spores in the place of a hyphal mat. These have an advantage over the above methodology in that only a couple of hyphae are present in the well plate for inductions, therefore any induction sites can be accurately identified, even when there is no apparent visible change to the hyphae.

Asexual zoospores of *A. bisexualis* were obtained by growing a mass of hyphae then starving them of nutrients. To accomplish this five plugs were taken from the margin of a colony of *A. bisexualis* growing on PYG agar, excess agar was removed, then the plugs were transferred to a sterile nappy liner (Treasures brand (Carter Holt Harvey tissue) boiled three times in distilled water to remove any anti-microbials) lined PYG agar plate. This was then allowed to grow for a period of 24 hours at 24°C in the dark, during which the hyphae grew through the loose weave of the nappy liner. This was then removed from the plate, taking the hyphae with it, and transferred to a flask containing 100mls sterile PYG broth, then incubated for 24 hours at 24°C in the dark with gentle shaking. The

hyphae were then rinsed with mineral salts solution (details of which are given in Appendix 1), four times during the first hour, then finally after another hour rinsed with sterile distilled water and left overnight in 100mls distilled water. This was carried out at 24°C with gentle shaking. Filtering of the resulting solution through a sterile Kimwipe removed the nappy liner and any loose hyphae from the released spores, thus giving a solution of *A. bisexualis* spores that were suspended in sterile distilled water. The spore solution was gathered into 10ml centrifuge tubes, vortexed for 10s at a medium setting, encysting the spores, then centrifuged at 800rpm for 15 minutes to concentrate the zoospore solution. Nine mls of the supernatant from each tube was carefully discarded using a pipette; the final ml was used to resuspend the pellet of spores, which were then collected in a sterile universal tube. A titre of around five thousand spores per 100 µl was normal for spores produced in this manner. Allowing an additional 12 hours to the incubation in distilled water boosted spore counts to as high as ten thousand spores per 100 µl, although at this concentration the spores grew too closely to each other to be used. Viable spores could be kept for up to two months at 4°C, although there was a gradual decline in viability with time. After a month of storage as little as fifty spores per 100 µl could be observed. For this reason, and because the necessary daily handling of the preparation led to frequent contamination, the spore preparation was carried out on a weekly basis during experimental work, to yield a constant supply of fresh spores. This also ensured that as far as was possible spores of a uniform physiological status were used between experiments.

Observation of Natural (i.e. Non Induced) Branching Patterns

For the induction experiments it was first necessary to observe the natural branching patterns of germinating spores in order to later confirm the action of induction mimicked the natural branching process. To accomplish this, spores were germinated overnight on a cellophane lined PYG plate at 25°C in the dark. The next morning, individual spores were cut out from the cellophane and transferred to cellophane lined 40mm Petri dishes containing PYG agar. The spores were then kept at 25 °C and observed and photographed every hour (at 15x magnification through a Wild M400 stereomicroscope and photographed with an attached Photometrics 1.3 Megapixel Coolsnap Cool Digital Camera). After an average time of 18 hours (n=50) growth the first branch could be observed in the spore, usually followed by a second after an additional hour. The linear spacing of these branches appeared random.

These results, unfortunately, could not be used for their intended purpose, as the procedure for the inductions had, at a later stage to be altered in order to use the spores. By germinating the spores on top of PYG agar (which is termed non-invasive growth) they were in no way representative of the spores used in the induction trials, which grew within and not on top of DMA (which is termed invasive growth). However, some relevant information on spore behaviour could be inferred from this first trial. Spores are known to branch less frequently on low nutrient media (such as DMA) compared to high nutrient media (such as PYG). It can be assumed, then, that as spores took 18 hours to branch on PYG, they would take at least this time to branch on DMA. An additional

lesson came in the mounting of the spores, 50% of germinated spores died in the course of the observations. The two possible causes of this were desiccation, as the spores were open to the air, and mechanical damage from bending the cellophane, and thus the spore, during transfer from the germination plate to the 40mm observation plate.

It was intended to repeat this initial trial after the initial inductions were completed, when the method of mounting the spores had been finalised. An unfortunate side effect of the difficulties encountered in mounting the spores, however, was that multiple slides would have to be set up, as cell mortality in the mounting stage was high and never fully resolved. Thus the spores used could be anything from 18 to 28 hours old, with the larger, older, branched spores offering higher survival rates. As the trial continued spores were grown for 24 hours at 25°C in the dark to allow germination before use, giving larger hyphae with multiple established branches, which were all candidate sites for the subsequent inductions.

Mounting

Using spores for the generation of the experimental hyphae neatly avoided the problem of overcrowding in the well plates but introduced several additional problems. Morris' method of mounting the experimental material (in Morris' study, hyphae, in the present study, spores) in low melting point agar was attempted but subsequently abandoned. Spores were originally germinated over a period of 24 hours on cellophane lined DMA plates. The germinated spores were incredibly fragile compared to the hyphal mat,

susceptible to mechanical damage incurred during peeling the spore-laden cellophane off the germination plate, desiccation, mechanical damage incurred as the germinated spores floated in the low melting point agar and finally heat damage from the low melting point agar, which could be cooled as far as 25°C while still remaining fluid. In twenty attempts to get the method to work, only one slide with successfully growing hyphae was obtained. It is thus likely that the methodology of Morris (1999) is only suitable for hyphal mats and not for spore preparations that appear to be more sensitive to stress.

The solution to this problem came in the invasive growth techniques developed by Walker (2004). Rather than applying the agar to the naked, fragile germinated spores, it was applied to the resistant, encysted spores before germination. Again, varying amounts of spore solution (typically 50, 100 and 200µl of spore solution would give isolated germinated spores on at least one plate) was applied to cellophane lined DMA plates, but then 800µl of low melting point 1% DMA agar was applied to the spores and allowed to set (typically for a period of five minutes). The spores were then left for 24h to germinate, protected inside the thin layer of DMA.

With the spores thus protected, it was relatively straightforward to then mount them in the well plates. Individual spores were identified under 20x magnification on a stereomicroscope (model as above), and then they were cut out of the plate, LMP DMA, spore and cellophane together. The thin layer of agar appeared to protect the spore from the most gentle mechanical disruption, desiccation and heat shock during the transfer to the well plate. The spores were then floated off the cellophane with 1ml of sterile DMA

broth; the cellophane was removed, then the DMA broth was wicked off using a folded Kimwipe. Finally the spore containing plug of DMA was sealed to the well plate by surrounding it with 50µl of LMP DMA and leaving it to set for two minutes, then covering the spores with 500µl of DMA broth. Usually five slides were made up in this way, and then the spores were left for at least half an hour for recovery after mounting. Approximately three out of every five growing spores ceased horizontal growth (i.e. growth that was parallel to the base of the slide) at this stage, growing upwards, out of the plane of focus, ultimately to grow non-invasively on the surface of the agar. Using lower density agar (1% low melting point) reduced the number growing non-invasively, but some hyphae were still observed growing vertically (i.e. perpendicular to the base of the slide). Only plates that had the spores growing horizontally were used for inductions, others were discarded.

Inductions

Hyphae were induced to branch by means of localised application of 1mM phenylalanine via a micropipette. Micropipettes were made from borosilicate glass capillaries (outer diameter 1.0mm, inner diameter 0.58mm with inner filament (Clark Electromedical Instruments, Reading, UK)) by a single pull from a Narishge PC10 (Japan) micropipette puller. 1½ weights were used with a temperature setting of 55°C to give a gently sloping tip with a bubble number of 24. Bubble numbers were obtained by attaching the micropipette to a 60cc syringe by a flexible tube. The micropipette tip was lowered into a beaker of methanol, and then the syringe was depressed until bubbles appeared from the

tip of the pipette. The amount of air remaining in the syringe was recorded; this value was taken as the bubble number. This gave an easy yet relatively accurate reading of the size of the opening in the micropipette tip.

Phenylalanine was used as the branch inducing agent. Micropipettes were filled with 1mM phenylalanine in DMA broth solution, and then inserted into a silicone oil-filled microinjector. A 5mm O-ring was cut from a length of silicon tubing to seal the micropipette to the injector, then fitted with the base of the micropipette protruding 1mm from the O-ring, this ensured some consistency in the pressure that was applied to the micropipette. Afterwards the pipette was screwed in tightly. This supplied enough pressure for the gentle release of the induction solution; tests with dye-filled micropipettes showed that the application of any additional pressure led to the hyphae being flooded with solution. This had the effect of poisoning the hyphae with excess phenylalanine and prevented any further growth. This was confirmed when application of 100µl of the induction solution to a prepared spore in a well plate led to the total cessation of growth. To prevent the over exposure to phenylalanine and damage to the experimental hyphae, whenever a new micropipette was prepared it was trailed on a separate hyphae, if branches were induced successfully then the experimental spores were induced. Finally the microinjector was attached to piezoelectric micromanipulators. These, combined with the shallow angle of the tip of the micropipette, enabled the micropipette to be placed adjacent to the growing spore with minimal disruption to the hyphae or surrounding agar. Inductions occurred when the micropipette was placed

directly touching the hyphae up to a distance of 30µm away from it. For consistency a distance of about 10µm was used for all trials.

A period of around eight minutes was necessary for a visible bump to form at the induction site (Figure 2.1), however the presence of the micropipette was necessary for continued growth. With the micropipette removed the bump ceased growth, if it was of a large enough size, or would appear to gradually retract into the hyphae over a period of half an hour if it was not. This apparent retraction was due to the enlargement of the hyphal diameter, which thereby encompassed the bump. After a period of 12 minutes exposure to the inducing micropipette a full branch would develop, which would continue to grow in the absence of the micropipette. All induction trials were carried out at a temperature of 20°C.

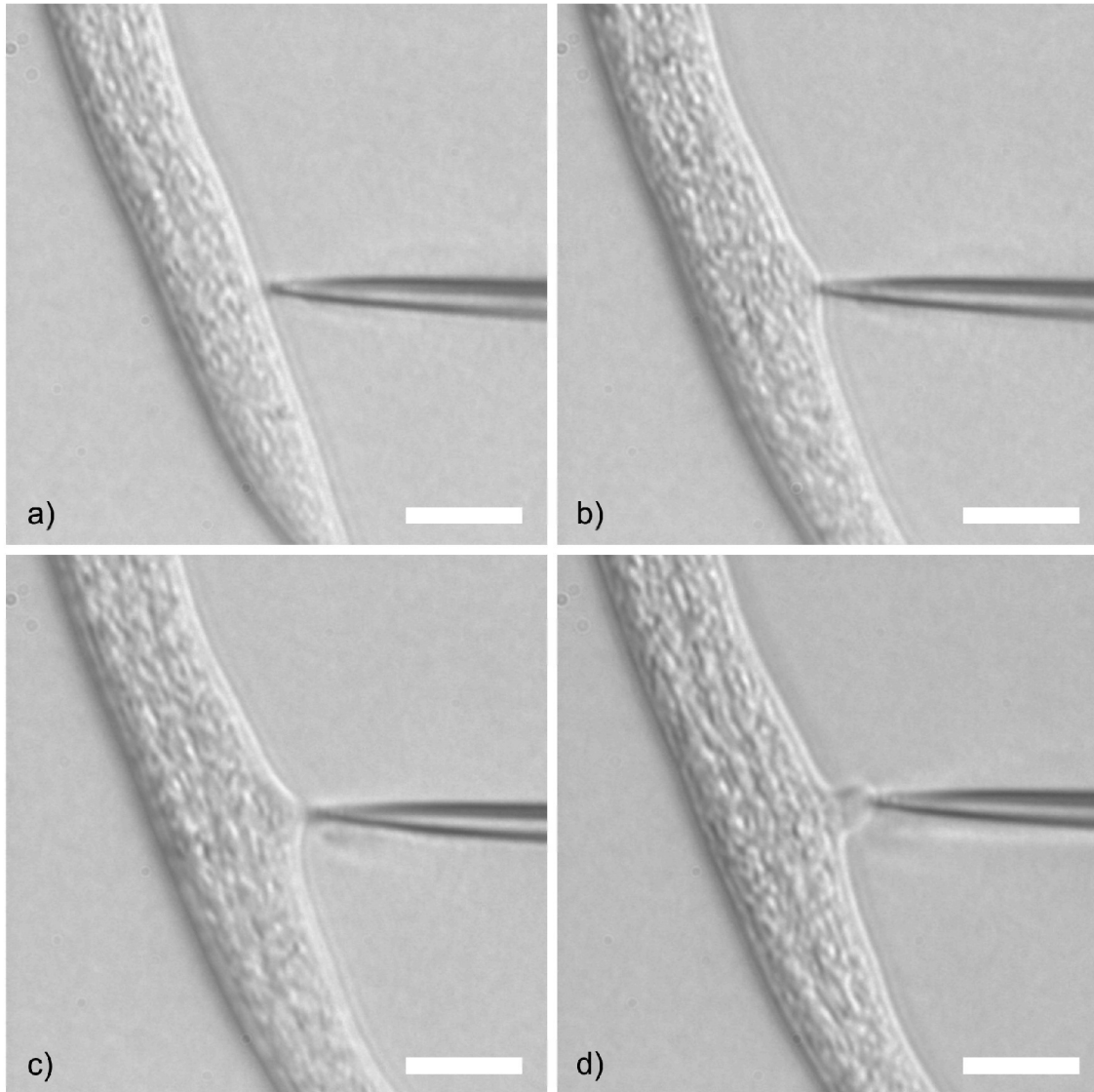


Figure 2.1. Induction of a branch from a growing hypha. a) $t = 0$, initial placement of micropipette. b) $t = 8$ min, bump emergence, first visible change in the hyphae. c) $t = 9$ min, bump enlargement. d) $t = 10$ min, full branch developed, branch growth now continues after removal of the inducing micropipette. Bar equals $10\mu\text{m}$

Fixation

For detailed visualisation of the actin cytoskeleton a mixture of 0.5% methylgloxal and 4% formaldehyde was used (Yu *et al*, 2004) (for details of this see Appendix 1). This gave a more permanent fixation than formaldehyde alone, allowing more detail to be captured in the visualisation. Well plates were flooded with 700µl of fixative, and then left in the dark for 45 minutes for the fixative to penetrate the agar to the hyphae. After this, the fixative was wicked off and the hyphae were rinsed five times with 700µl of PIPES buffer, pH 6.8, with each rinse lasting five minutes.

To confirm the value of the inclusion of methylgloxal to the fixative, additional experiments were carried out in which germinated spores were mounted in well plate slides as above but then fixed with formaldehyde alone. The results of this showed negligible background fluorescence but with a marked loss of contrast in the images. While an actin deplete zone could be observed after staining in one formaldehyde fixed tip the edge around the zone was hard to distinguish (Figure 2.2.). It was thus decided that the methylgloxal and formaldehyde mix gave a better preservation of the F-actin cytoskeleton in the cells (for representative images using the combination fixation see subsequent chapters). This finding is in agreement with those of Yu *et al*. (2003). Therefore the combination fixative was used in all subsequent induction trials.

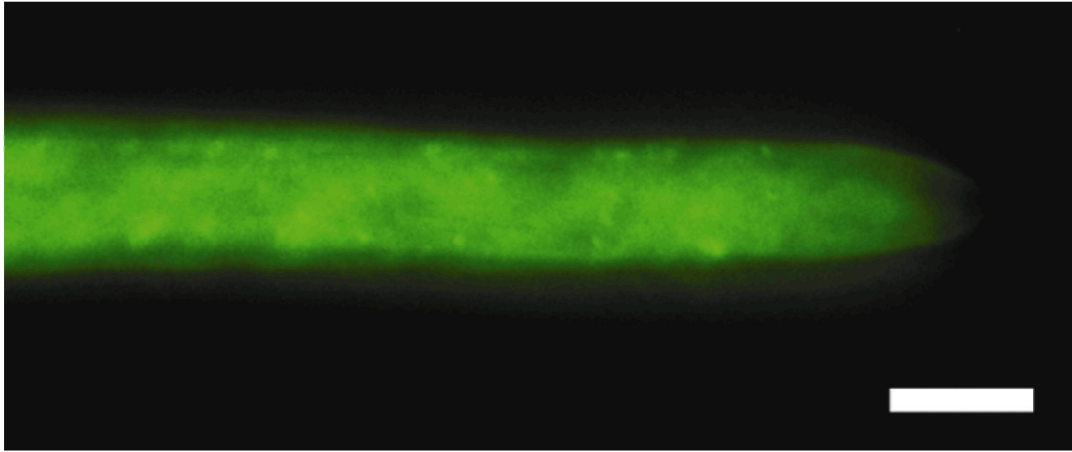


Figure 2.2. An *A. bisexualis* hypha that had been fixed with formaldehyde solution alone and then stained with Alexa Phalloidin. While an F-actin deplete zone is visible, it is undefined around the edges. Bar equals 10 μ m

Staining

Alexa Phalloidin, an actin binding stain, was used to visualise the actin present in the induced branches. Immediately following fixation, the hyphae were flooded with 30µl Alexa phalloidin solution (stock obtained from Molecular Probes and diluted 1:4 with 50 mM PIPES) and left in the dark for 45 minutes for staining. After this time the cells were rinsed by attaching the well plates to a Petri dish by tape and flooding the slide with 20mls of PIPES buffer, then placing the assembly on a gentle shaker in the dark for ten minutes. This was repeated four times, then the slides were drained and excess buffer was wicked off. Finally, a drop of anti fading solution (*p*-phenylenediamine made up to 0.1% in distilled water) was added to the slide, after which the spores were imaged.

Imaging

Originally, it was planned to observe the F-actin cytoskeleton by means of confocal microscopy. This had provided high quality images, in conjunction with the aforementioned fixing and staining procedures, in previous studies of the F-actin cytoskeleton in hyphal tips of growing *A. bisexualis*. Unfortunately the necessity of a large layer of agar to protect the spores meant that the confocal laser couldn't supply enough light for high quality imaging. This led to the use of an Olympus IX70 inverted microscope for epifluorescent microscopy, which acted upon the same stains as the confocal laser would have, but provided enough light for detailed images to be captured through a 40 times oil immersion objective by a Photometrics 1.3 Megapixel Coolsnap Cool Digital Camera. All images of the cytoskeleton were obtained in this way.

Latrunculin B Concentration tests

The second part of the research sought to further investigate the role of F-actin in branch formation by adding an actin inhibitor, Latrunculin B (Lat B), to the induction micropipette. First tests of the effects of various Lat B concentrations were undertaken. Five well plates containing spores were prepared, as described previously for the induction trials, each of these was observed for 30 min to confirm normal growth prior to the addition of Lat B. Five different concentrations of Lat B were used; 0.5 $\mu\text{g/ml}$ ($=1.25\mu\text{M}$) (Bachewich and Heath, 1998), 1.0 μM , 0.5 μM , 0.25 μM and 0.1 μM (Dean, 2004), all of which were made up in DMA broth. Five hundred μL of inhibitor in DMA was applied to each well plate, flooding the spores with inhibitor solution. Initially spores were left in the dark, for a period of 15 minutes, a time equal to the longest time a spore had been exposed to the induction micropipette in the previous trial. Spores were then fixed, stained and observed as described above. The fifteen-minute exposure led to disruption of the F-actin cytoskeleton in all slides except the 0.1 μM treatment, however the effects were somewhat variable. It was assumed that this variability was due to uneven distribution of the inhibitor solution through the surrounding agar, therefore a longer time of exposure was used to try to enable a more even distribution. In the fixing and staining procedures, 45 minutes exposure to the respective solutions gave thorough distribution of solutions through the agar, so a 45 minute exposure was selected for the Lat B concentration trials. With this exposure, the agar was thoroughly penetrated by the solution, leading to an even, total disruption of the F-actin cytoskeleton at all concentrations, apart from 0.1 μM , which showed variable disruption to the F-actin in the

cells. Based on these tests, a concentration of 0.25 μ M Lat B was selected for the subsequent Lat B induction trials

Inductions, with Latrunculin B

Spores were prepared as for the previous induction trials and again mounted in well plate slides with LMP DMA. Induction solutions were prepared as before with the addition of 0.25 μ M Lat B and filled pipettes were fitted to the micromanipulators and brought next to the cell. When branches formed normally with the added Lat B it was assumed that the concentration of Lat B used was insufficient to disrupt the F-actin filaments during the 15 minutes of exposure to the micropipette; branches induced continued growing as normal. A higher concentration of Lat B, 2.5 μ M, was selected for the repeats of the experiment. The reasoning for this selection was that, as the concentration of phenylalanine successfully used in the previous induction trials was poisonous when applied to the whole plate, stopping all growth, so a much higher than needed concentration of Lat B may be needed for any disruption of the actin cytoskeleton. 2.5 μ M, twice the concentration used by Bachewich and Heath (1998), was selected for this reason, and all subsequent inductions with the inhibitor were conducted using this concentration. After induction, all cells were fixed, stained and observed as before.

Chapter 3

Induction trials

Introduction

As detailed in Chapter 1 the F-actin cytoskeleton is thought to play an essential role in both hyphal tip growth and in hyphal branching. With respect to the latter process studies have thus far been limited due to the inability to accurately predict the timing of the process. Most of the information known about hyphal branching has been extrapolated from studies of hyphal tip growth. Heath *et al.* (2000) showed that the F-actin cytoskeleton was essential to hyphal tip growth, the same study showing that disruption of the cellular microtubule network had a minimal effect on the rate of tip growth. As the process of hyphal branching is essentially the formation of a new growing tip, it stands to reason that actin would be similarly be as central to the branching process as it is to tip growth.

Bachewich and Heath (1998) utilised the hyperbranching form of *Saprolegnia ferax* to study changes in the F-actin cytoskeleton during the localised polarisation leading up to branch emergence. In regions that showed increased branching with longer incubation, the formation of radial F-actin arrays were observed prior to any detectable surface protrusions. It was hypothesised that these arrays could perform a role in vesicle delivery to the branch site, softening the cell wall at the branch site, as in the turgor driven mode of growth, or they may exert mechanical stress on the branch site, physically pushing the

branch out. Radial F-actin arrays were also observed proceeding germ tube emergence in germinating asexual spores, confirming their role in the localised initiation of polarised growth. It was intended to study these pre-bump events as part of this thesis, however as described in the preceding chapter it was not feasible to do so with the experimental methods utilised.

With the development of branching induction techniques it is now possible to more accurately determine when a branch is going to form. Furthermore the development of improved techniques for chemical fixation of hyphae means that it is now possible to preserve and thus observe new structural aspects of the F-actin cytoskeleton.

In the model proposed by Jackson *et al* (2001), there are two possibilities of what might occur during bump formation. Bumps are the progression from the radial F-actin array of the transduced branching cue to the F-actin cap, (which in invasive hyphae may also contain an apical F-actin deplete zone) of a growing hyphal tip. Thus the bump must either result from the development of the F-actin cap, evident from a higher density of actin at the branch site or the development of the deplete zone, which would be evident from a decrease in staining intensity. In the former of these scenarios the F-actin cap may provide a protrusive force which results in bump formation. With the second possibility an F-actin depleted zone could provide less resistive force to turgor pressure, thus facilitating greater yielding of the wall in a subapical location that again will result in the formation of a bump.

Post bump formation, what might be expected with respect to branch development is much clearer, due to the previous research into the process of tip growth. Growing oomycete tips contain an F-actin cap and, in invasive growth conditions, an apical F-actin deplete zone. The emergence of these two structures can thus be expected during the transition from bump to branch proper, if they hadn't already formed in the bump stage. Heath (1995) had previously described F-actin caps in emerging branches, however this research was conducted on hyphae that had been fixed with formaldehyde, therefore any F-actin deplete zones in the branch tips may have been lost in the process of fixation.

Materials and Methods

Materials and methods were as described in Chapter 2.

Results and Discussion

The original plan was to obtain a time series of images of the growing branch, from initiation through to branch emergence. Attempts at observations of pre-bump structures and F-actin staining patterns were initially trialled but subsequently abandoned, as very early bump stage inductions were lost as the hyphal width increased. Thus it was not possible to accurately determine the location of, and thus be able to capture images of, an induced pre-bump structure. For this reason additional attention was given to the bump stage, the majority of inductions performed focused on this stage of branch formation, as it was the earliest stage that could be reliably observed.

It is likely that after cue transduction, but prior to branch formation, any radial F-actin array will be assembled. However, between the formation of the array and the emergence of the branch proper, in the bump stage of the branching process, there is little detail of what to expect. As a F-actin cap can be observed growing hyphal tips, its development must occur during the bump stage of branch formation, with an actin deplete zone emerging at some stage. This was another reason why inductions performed focussed on the bump and early branch stages.

The second reason for focussing on the bump stage of branch formation was one of technique. Smaller bumps were often “reabsorbed” by the growing hypha as it expanded, and induced full branches frequently ceased lateral growth, growing vertically and thus out of the microscopes field of view. These two complications meant that early bumps and full branches could only be induced immediately before fixation in order to be able to image them. Larger bumps, however, could be induced up to an hour before fixation and would maintain their shape for that time, while other inductions could be performed in the interim.

Confocal microscopy of induced branches proved impractical, due to the problem that the layer of LMP DMA agar that was necessary to protect the spores was found to absorb most of the lasers light. Less agar allowed confocal use, but decreased the already low survival rate of spores. Therefore the detailed composite images of the stained actin cytoskeleton, with both filaments and plaques visible, could not be obtained.

Epifluorescent microscopy allowed the use of thicker agar, giving higher spore survival rates in preparation, but did not facilitate composite images.

F-actin deplete zones were most apparent at two stages of the branching process. Firstly deplete zones were apparent in the earliest phases of bump emergence, before the bump had developed to its full size. Thus after the earliest stages of bump formation, with an F-actin deplete state, reorganisation of the actin cytoskeleton must take place at the bump site. From full bump through the transition to branch proper the deplete zone disappears; through these stages the developing branch is filled with actin plaques and microfilaments. Finally, after the bump stages and the transition to the branch proper an F-actin deplete zone re-emerges in the new growing tip. At this stage the new branch is fully established and is largely independent of the parental hyphae (Table 1, Figure 3.1).

Comparison of Induced Branch State and Size

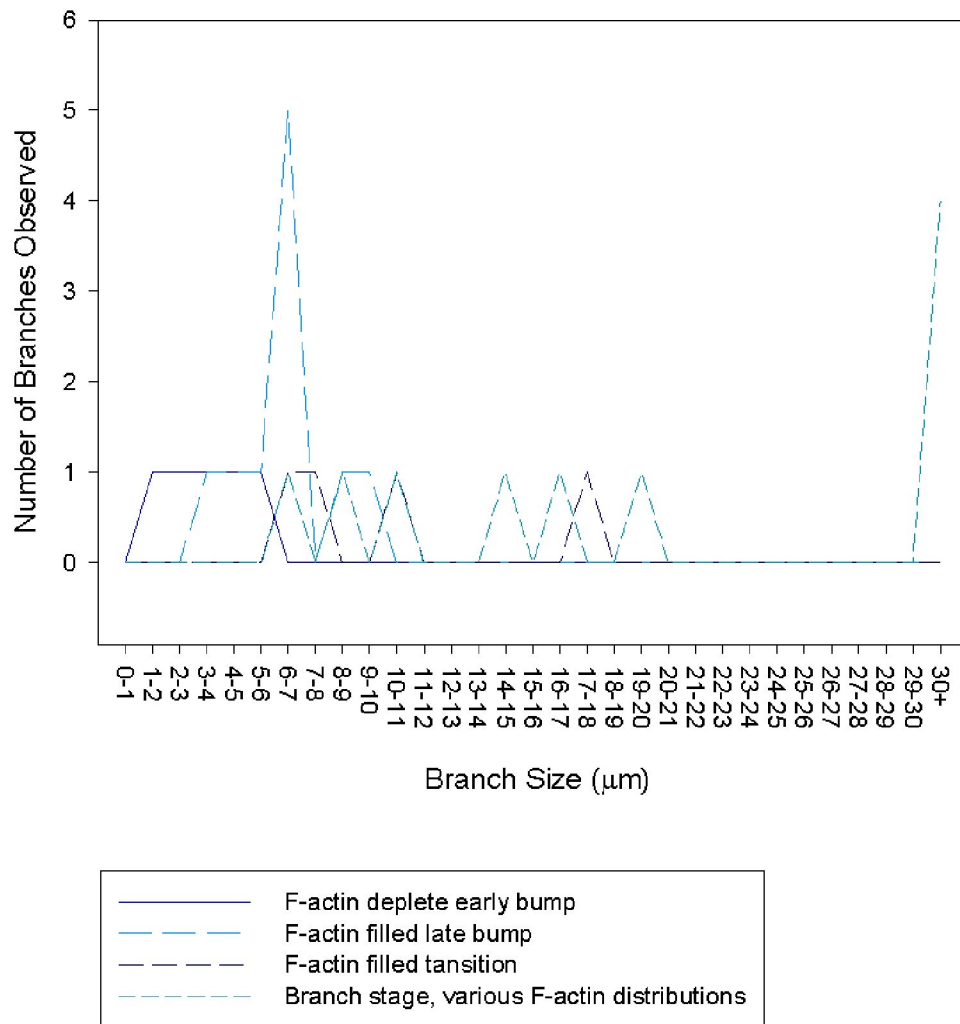


Table 3.1. Observed results of the induction trials. Results are arranged by the stage in which they occur, determined by the developing branches shape and F-actin distribution. In the case of the full branches, 50% observed showed apical F-actin deplete zones, with no clear pattern as to their development.

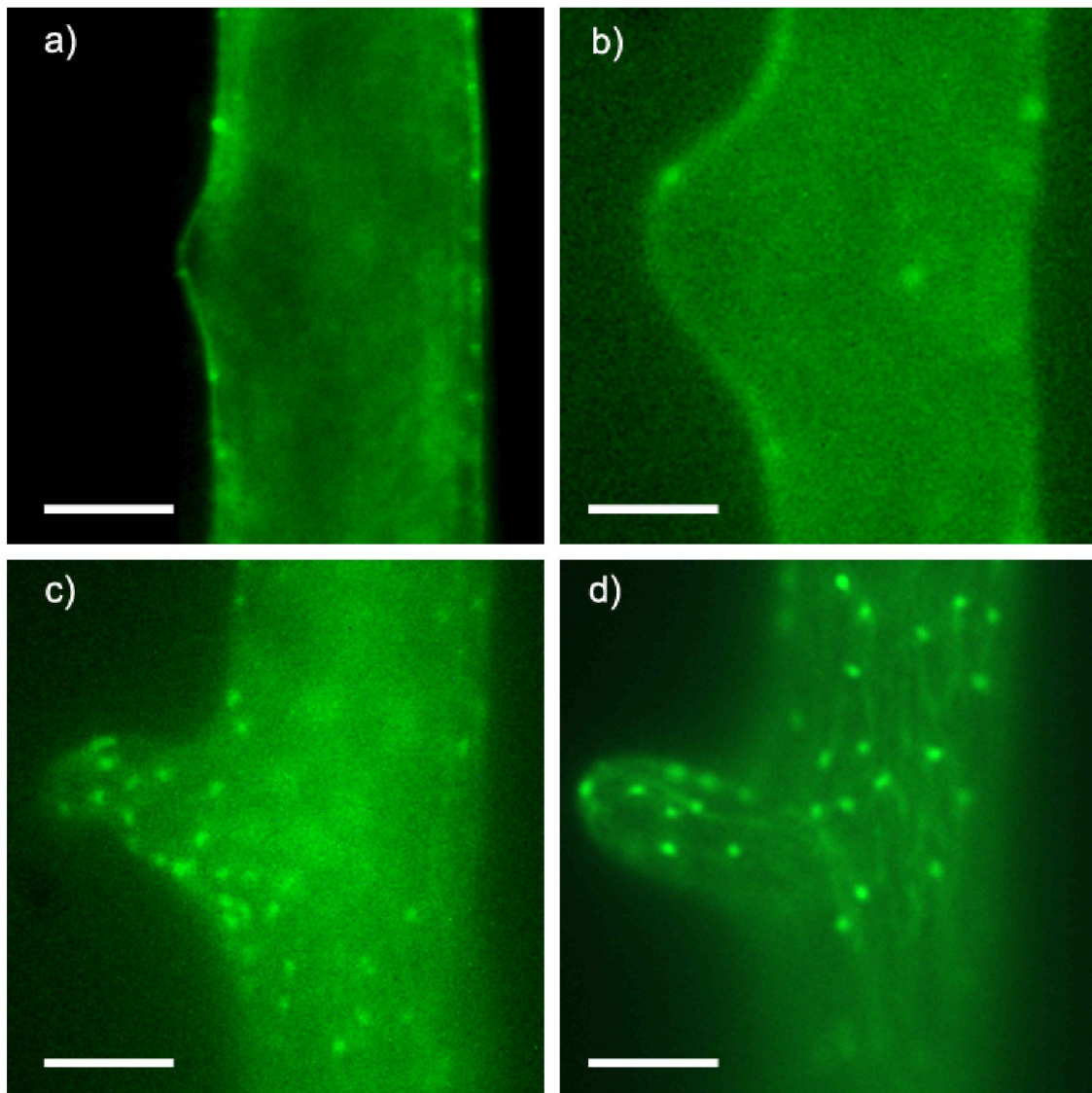


Figure 3.1. The four stages of branch development. a) Early bump. Note the F-actin deplete zone. b) Late bump. The bump fills with F-actin. c) Transition from bump to branch. d) Emergence of the branch proper, with F-actin cap. Bar equals 5 μ m.

During the induction trials an induction was attempted in which the micropipette punctured the cell. The cell self repaired the puncture, leaving the hyphae to continue growth. As the same well plate contained other growing spores, each with multiple successful inductions on their hyphae, the slide was fixed and stained as usual. The punctured cell was then examined under the epifluorescent microscope along with the other induced experimental hyphae on the slide. This revealed the patchwork of actin around the wound, showing its structure clearly (figure 3.2). While this observation has only been observed on the one occasion it is of interest in light of the findings of Levina *et al* (1998), where the F-actin cytoskeleton was tied to the wound response. Levina *et al.* (1998) found that a damaged hyphae' ability to repair itself was impaired, although not eliminated, by the action of F-actin inhibitors on the punctured hyphae.

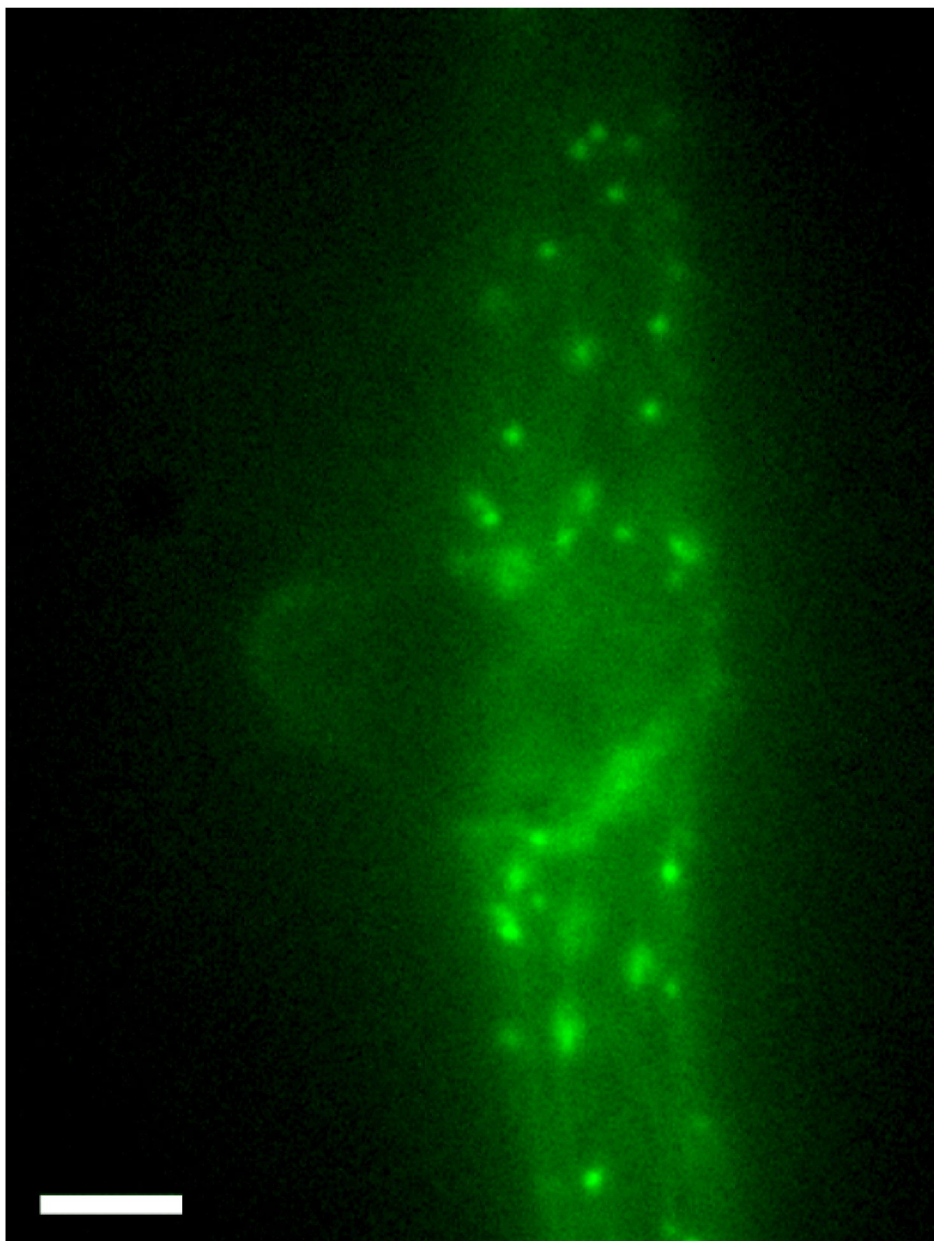


Figure 3.2. Alexa Phalloidin stained hyphae following inadvertent puncture of the cell during the course of the induction. Dispersal of cellular contents following the wounding of the cell resulted in the diffuse staining surrounding the cell. Note clumped F-actin in the sealing plug. Bar equals 10 μ m

In light of the above findings it is possible to speculate as to the cytoskeletal dynamics and how these relate to the model of Jackson *et al.* (2001). The apparent retraction of early-stage induced bumps, caused by subapical thickening of hyphae, when the inducing agent is removed suggests that the original model be modified to incorporate this fact; the bump stage should thus be divided into two distinct stages. In the early bump stage the cell has not committed itself to the formation of the branch, removal of the inducing agent leads to the cessation of growth of the new branch, which is then lost as the hyphal width thickens. The early bump is represented by an F-actin deplete zone in the area protruding from the parental cell wall, it is hypothesised that this deplete zone occurs because the early bump is purely turgor driven, occurring due to a localised weakening of the cell wall and less resistance to turgor due to the localised absence of F-actin. From this it can be concluded that major rearrangement of the actin cytoskeleton has not yet occurred at this stage.

The second stage of bump formation, the late bump stage, is associated with the rearrangement of the F-actin cytoskeleton into the new branch. By this stage the cell has committed to the creation of the new branch; removal of the inducing agent results in continued normal growth and the development of a full branch. In the late bump the actin deplete zone disappears; the bump is filled fully with plaques and filaments, as it remains during the transition from late bump to the branch proper. Only once the new branch is fully formed does the apical F-actin deplete zone develop again.

There is a brief transition state between the two phases of bump development. This “mid bump” stage occurs with the development of the new branches cytoskeleton, when the new bump is moving from the actin-deplete state to one with its full actin framework. Removal of the inducing agent at this stage leads to the cessation of growth but not the reabsorption of the new branch. Bumps can be left in this state for an hour without any change in the outward appearance of the bump.

The conclusion of this research suggested that the actin cytoskeleton was not necessarily necessary for bump emergence, however, for the progression from bump to branch localised reorganisation of the actin cytoskeleton was essential. This theory was tested by the subsequent inhibitor trials, with the addition of Latrunculin B, a potent inhibitor of filamentous actin, to the inducing solution.

Chapter 4

Latrunculin B Induction Series

Introduction

As detailed in Chapters 1 and 3 F-actin has previously been thought to play a key role in the process of hyphal branching. In concert with this, in the previous chapter the induction trials showed that from the mid bump stage onwards the emerging branch contained a fully developed actin cytoskeleton. According to the model of Jackson *et al* disruption of the actin in the emerging branch would be expected to lead to the total cessation of branching at that site. In order to test this hypothesis, a series of inductions were undertaken in the presence of the F-actin inhibitor Latrunculin B.

The action of Lat B has been shown to disrupt the F-actin cytoskeleton, and in the process interrupt hyphal growth, in a number of related studies. When growing *Saprolegnia ferax* tips were treated with the inhibitor, the observed results varied according to the mode of growth of the cells (Gupta and Heath, 1997). Under high turgor conditions cells treated with Lat B showed a brief period of increased growth, consistent with the theory that the actin cytoskeleton plays a role in restraining the growing tip. In contrast to this, treated cells under low turgor conditions showed decreased growth immediately after treatment, consistent with the amoeboid growth model. This finding gave additional weight to the proposal that both modes of growth occurred in the same

species depending on growth conditions. After 60s of exposure to Lat B all cells ceased growth, as shown in Heath *et al.* (2000).

Varvarigos *et al.* (2004) demonstrated the action of Lat B against branching in protoplasts derived from gametophytes of the alga *Macrocystis pyrifera*. Treatment with Lat B led to the complete disruption of the pre-branch radial F-actin arrays and the cessation of branching in the cells. While this finding corroborates with the expected results of this thesis, the marked difference in the model systems used means the same pattern of results may not necessarily be observed in the invasively growing oomycete model used in this thesis.

In the present study Latrunculin B, the most commonly used inhibitor of actin microfilament formation, was added to the induction solution in order to cause a localised disruption of the actin cytoskeleton at the branch site. Its effects on both branching and F-actin distribution were monitored.

Materials and Methods

Materials and methods were as described in Chapter 2.

Results and Discussion

As with the much of the previous experimentation on the subject of hyphal branching, to date work with Lat B has been limited to exposure to whole plate cultures. For this reason no suitable concentration data was available for micropipette application. Due to time limitations the construction of a concentration trial series using the micropipette was not possible. Therefore, as a compromise, varying concentrations of Lat B were tested on spores mounted as in the induction trials, but the Lat B solution was applied to the whole well plate instead of the intended micropipette application. The spores were then fixed and stained as before to observe any disruption to the actin cytoskeleton.

Latrunculin B is believed to act by complexing available G-actin in the cytoplasm thus preventing its polymerisation into filaments (Schaten *et al.*, 1986). The action of LatB proved reversible. Removal of the micropipette would lead to reversion to the normal growth of the hyphae. This was most dramatically demonstrated when the inhibitor laden micropipette was placed adjacent to the growing tip, in the region of the actin cap. The presence of the inhibitor resulted in the tip morphology changing markedly over a period of ten minutes, the normal parabolic curve shape of the normal hyphal tip replaced with an elongated, sharply pointed profile (Fig.4.1). Over a period of 30 minutes, however, the normal tip profile was restored and tip growth resumed.

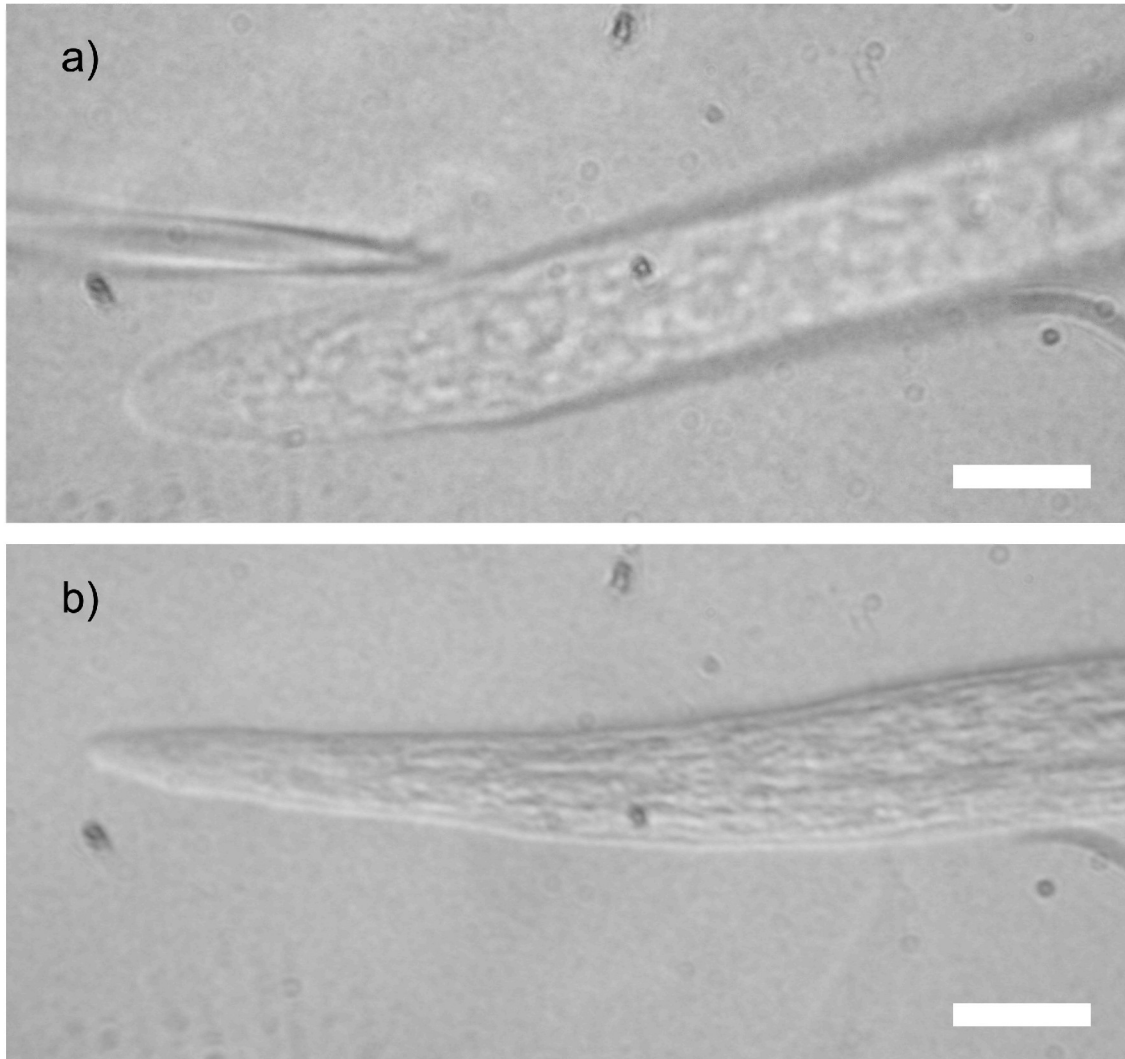


Figure 4.1. Comparison of the normal “parabolic” curve of a growing *A. bisexualis* tip with the “wedge” profile that accompanied localised inhibition of the apical actin cap. a) $t = 0$ min, placement of the inhibitor micropipette adjacent to the growing tip. b) $t = 12$ min, removal of the micropipette. Note chemotrophic growth of the tip towards the localised concentration of phenylalanine where the micropipette was. Bar equals 10µm

From the original model of hyphal branching the presence of actin inhibitors in the induction solution would be expected to completely prevent the formation of a branch. However, the results of the induction trials suggested that major rearrangement of the F-actin cytoskeleton might not be necessary for the formation of the early bump stage of branching. It was thus hypothesised that the inductions performed in the presence of the inhibitor may result in the formation of a small bump, but the inhibitor would prevent the formation of a full branch due to the impossibility of building a working actin framework with the dissolution of all microfilaments.

A final complication of this series of experiments was that while Lat B has been proven to completely disrupt cortical actin, it has little effect on the formation of peripheral actin plaques (Heath *et. al.*, 2003). With actin polymerisation into filaments disrupted by the localised application of inhibitor, the free monomeric actin released by this could accumulate to form an increased number of smaller actin plaques in the hyphae, away from the site of inhibition. The whole plate applications of Lat B in the concentration tests showed this pattern, even at Lat B concentrations that were too low to completely break up all the microfilaments in the hyphae.

An unexpected observation in the inhibitor trials was the relative normality with which branches were induced. Initial inductions used a Lat B concentration of 0.25 μ M, which was the most dilute concentration that had previously been shown to completely disrupt the actin microfilaments in the cell (Fig. 4.2) (see Chapter 2). With this concentration of Lat B mixed into the inducing solution (Appendix 1) bumps and full branches could be

induced normally, cells took around ten minutes for bump emergence compared to eight in the absence of the inhibitor. In view of this the experiment was repeated with a higher concentration of Lat B to ensure inhibition.

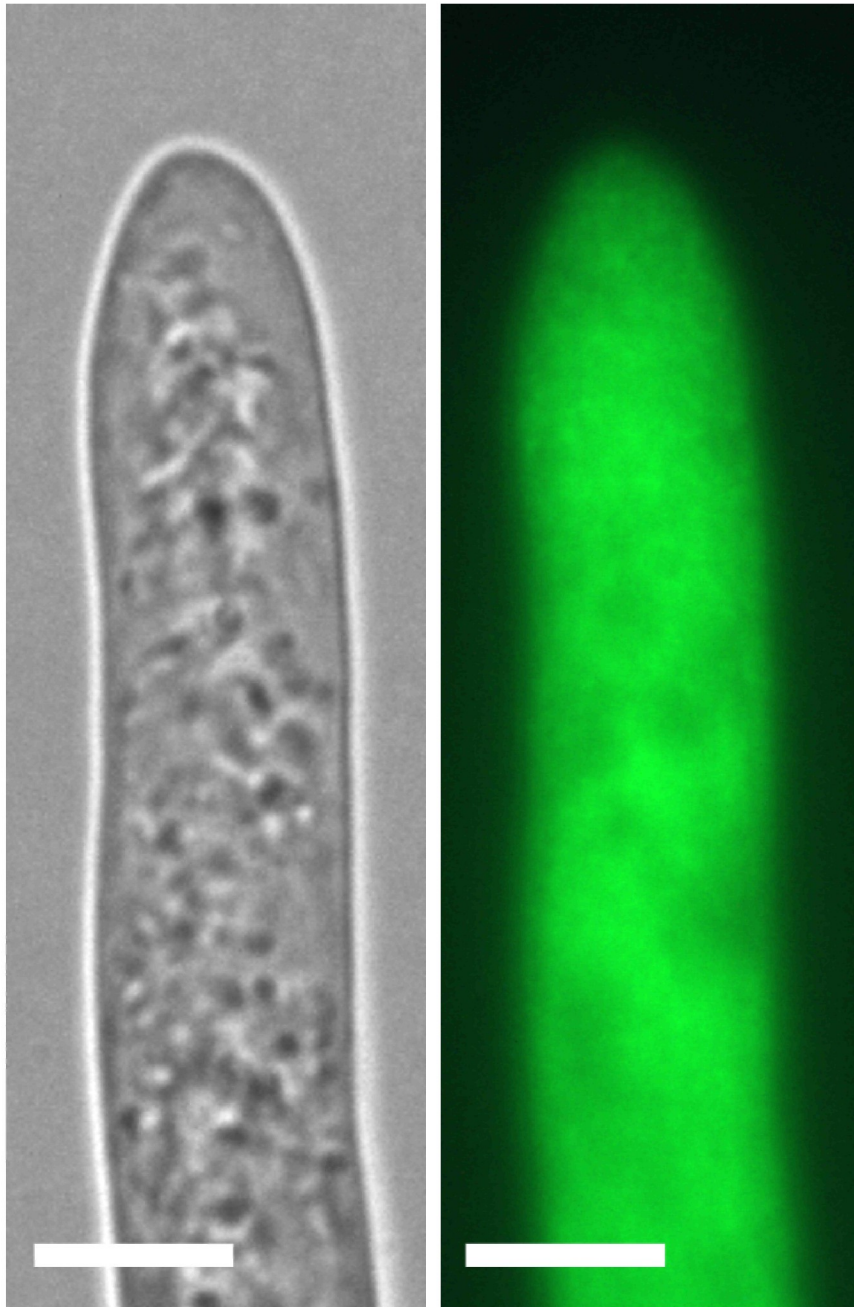


Figure 4.2. DIC and epifluorescent images of an Alexa Phalloidin stained hyphal tip after whole-plate treatment with 0.25mM Lat B. Note the complete disruption of the F-actin cytoskeleton in the cell. Scale bar equals 10 μ m

The increase in inhibitor concentration to 2.5 mM, however, did not result in the cessation of branching in response to the inductions as bumps and branches were observed with the higher inhibitor concentration. Again there was an increased time for bump emergence, as long as twelve minutes for the first visible change in the cell wall. These cells were fixed and stained as described previously to visualise the F-actin cytoskeleton.

As with previous experiments, precedence was given to induced bumps. The initial inductions series suggested that actin might not be strictly necessary for the emergence of the early bump; therefore the emergence of small bumps in the presence of Lat B was not surprising. The emergence of what initially appeared to be late stage bumps was unexpected, however. Alexa Phalloidin staining of the induced bumps (Fig. 4.3) showed them to be completely devoid of actin, therefore in terms of their F-actin distribution these can be regarded as still in the early bump stage, despite their outward appearance. These large, F-actin deplete bumps showed a rough clumping of actin around the base of the bump, similar to that seen in the punctured cell in the previous chapter. It is hypothesised that this clumping represents the edge of the effective spread of the inhibitor.

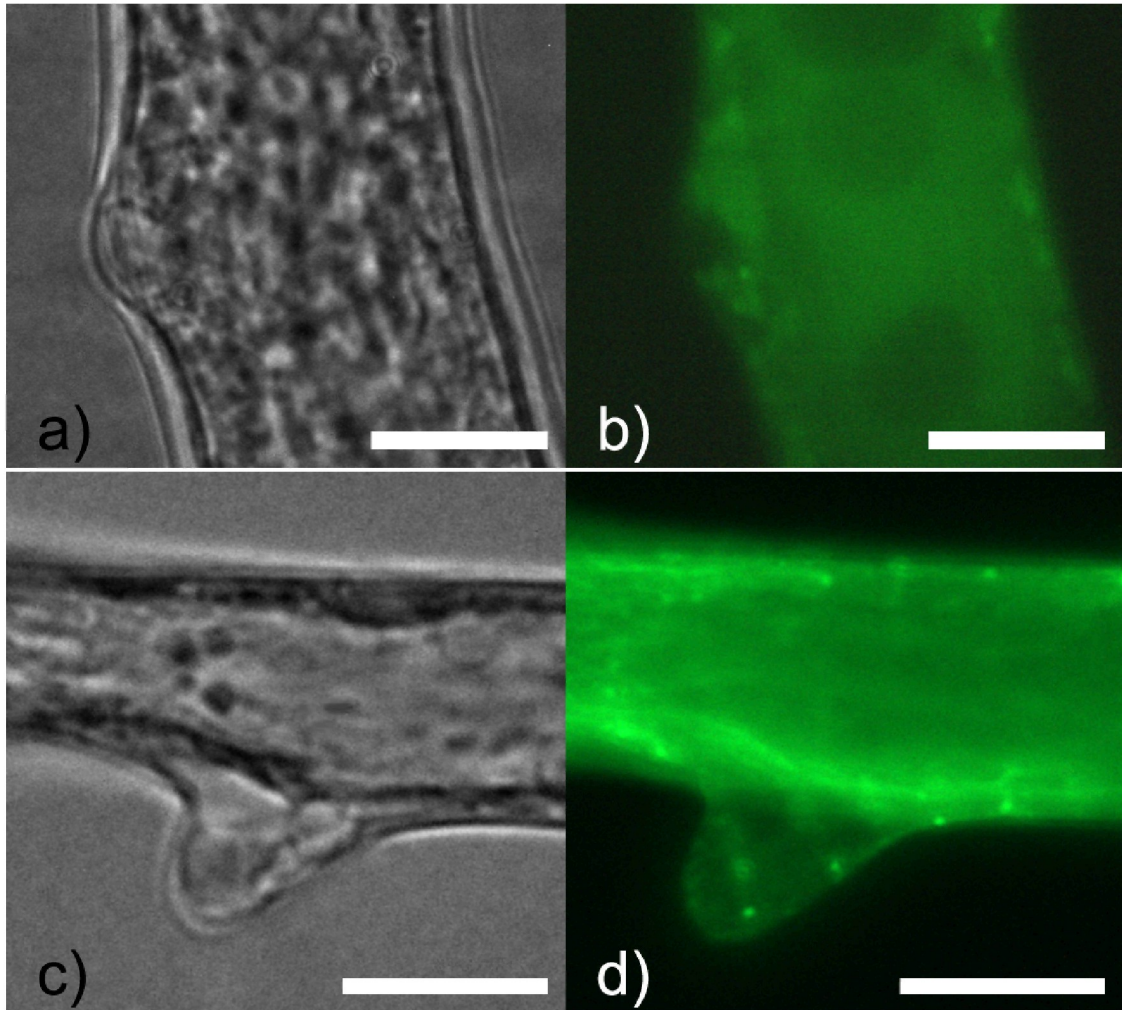


Figure 4.3. Bump stage inductions with the addition of 2.5 μ M Lat B. a) An induced bump outwardly still in the early bump stage. b) Alexa phalloidin staining shows an actin deplete zone in the bump, as expected in an early stage bump, although the F-actin at the bump base appears jagged and damaged. c) Another induced bump, outwardly in the late bump stage, prior to the transition to the branch proper. d) The same bump stained with alexa phalloidin, showing the induced bump to still be in the early bump stage, despite its outward appearance. Again, there is a build-up of f-actin around the base of the bump. Note F-actin plaque formation is unaffected by the action of Lat B. Bar equals 10 μ m

The main surprise of the inhibitor trials was the appearance of full branches in the presence of inhibitor (figure 4.4). Staining of these cells showed a damaged, patchy F-actin cytoskeleton, but the presence of actin at all was unexpected. While images were somewhat blurry, distinct actin plaques and microfilaments could still be observed in the induced branches.

This presents an interesting dichotomy of results, the induced bumps showing successful inhibition while the induced branches still retaining a damaged but functional actin cytoskeleton. A possible explanation for this centres around the differing methods of induction required for bumps and branches. In inducing bumps the micropipette remains stationary. This could in theory allow an applied concentration of Lat B inadequate for complete inhibition to pool and concentrate at the induction site, resulting in complete inhibition of actin polymerisation in the bump and the formation of an actin deplete zone. In the case of inducing full branches, however, the inducing micropipette is constantly pulled away from the parental hyphae, in order to keep the new branch growing in the same plane of focus. This constant motion could lead to the inhibitor becoming much more dispersed than in the bump inductions, resulting in a much lower applied concentration of inhibitor. When this is coupled with the fact that the action of Lat B proved reversible, as in the growing hyphal tip example given above, it gives a plausible explanation for the dual conflicting results of this study.

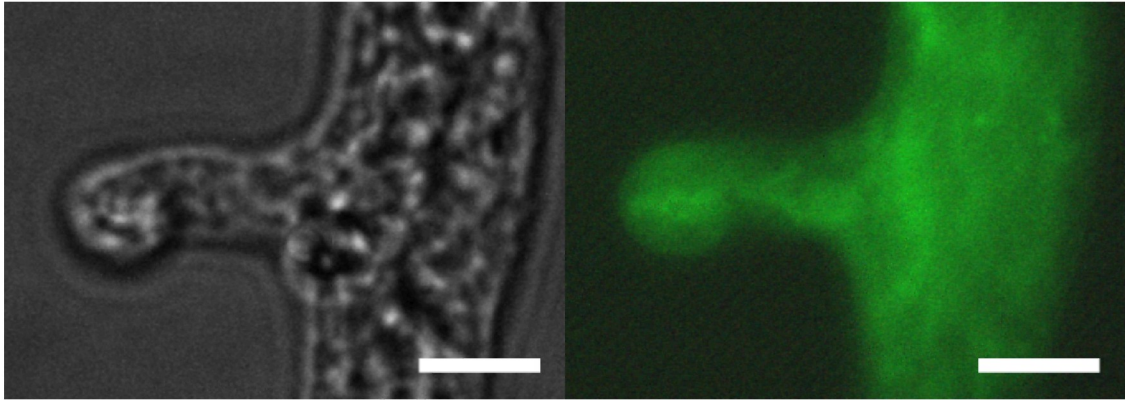


Figure 4.4. DIC and epifluorescent images of a branch induced with the addition of 2.5mM Lat B. The presence of F-actin microfilaments inside the branch suggests inhibition was less effective with the moving micropipette necessary for branch induction. Bar equals 5 μ m

Conclusions of the Inhibitor Trials

Deriving conclusions from the LatB trials is difficult, due to the incomplete inhibition of the induced full branches. However, valuable information can be taken from these experiments. The fact that bumps could be successfully induced in the presence of actin inhibitors adds credence to the theory that the early bump stage is turgor driven and thus relatively F-actin independent. Bumps could be successfully induced to outwardly appear to be in the late bump stage, but once stained the F-actin structure was the same as that of the early bump.

The previous chapter concluded that full branch emergence was linked with rearrangement of the F-actin cytoskeleton into the new branch. The successful induction of full branches in the presence of LatB at first appeared to contradict this finding. While the appearance of bumps in the presence of inhibitor was almost expected, the emergence of full branches came somewhat as a surprise. Staining of the F-actin cytoskeleton, however, showed that these induced branches did contain F-actin, this despite the application of inhibitor. This cellular F-actin was possibly damaged by the Lat B, despite the fact that blurry images from the final trial made interpretation difficult, still F-actin plaques and microfilaments could be observed in the branches. A repeat of this trial, with a higher concentration of LatB, would be necessary to confirm the results. Unfortunately due to time constraints this was not possible.

Chapter 5

Conclusions and Future Directions of Research

Conclusions

While hyphal tip growth is well studied, to date the process of hyphal branching is less well understood, mostly due to the unpredictable timing and location of branch emergence along a hypha. By combining previous work on branch induction with the existing studies of hyphal tip growth a working model of the process of hyphal branching was created. This model consisted of the reception of a branching cue, which was then transduced into a response. The first outwardly visible change in the hyphae was the emergence of a bump in the cell wall. This bump enlarged and eventually developed into a new branch. The model placed considerable emphasis on the F-actin cytoskeleton, and it was that which this thesis aimed to test.

This research firstly used methods of chemical induction to create controlled branches along hyphae obtained from spores of the oomycete *Achlya bisexualis*. Branches were induced for varying times to obtain a time series of branch development then fixed and stained for the F-actin cytoskeleton. The results of these experiments suggest that the process of bump emergence is in fact a two-stage process. Firstly, the localised thinning of the cell wall at the branch site leads to a turgor driven bulge in the cell wall. This early bump is actin deplete, as it occurs before the major restructuring of the F-actin cytoskeleton into the branch. The second stage of bump development is accompanied by

the construction of an F-actin network inside the new bump. It is hypothesised that this restructuring of the F-actin may mechanically push the new branch outwards, create a means for vesicle delivery to the new tip and/or act to regulate yielding of the new tip to turgor pressure. The late bump progresses through a transitional state before becoming a full branch. At this stage an apical F-actin deplete zone appears at the tip of the new branch and the branching process is complete.

This suggested model was tested through the addition of latrunculin B, an inhibitor of F-actin, to the inducing micropipette. Again, bumps could be induced, outwardly appearing to be in the late bump stage of development. Staining for F-actin and imaging however showed that these bumps were in fact F-actin deplete, suggesting that they were in fact still in the turgor-driven early bump stage. While the emergence of early bumps was to be expected, the previous experiment suggesting these were turgor driven and relatively independent of F-actin, the successful induction of full branches in the presence of inhibitor was an unexpected result. Staining of these induced branches showed a damaged but, it can be assumed, functional F-actin cytoskeleton, suggesting that the localised concentration of inhibitor may have been inadequate in these trials. Unfortunately time did not allow repeats of this experiment for definitive conclusions to be made.

Future Research

Actin Depolymerising Factor Staining

As it was hypothesised that the F-actin cytoskeleton at the branch site is in a constant state of rearrangement, it was originally intended to conclude this research by staining the induced branches with antibodies specific to cofilin/Actin Depolymerising Factor (ADF). A high state of flux in the actin cytoskeleton would be associated with an increased localised concentration of cofilin/ADF at the branch site. Cofilin/ADF has been shown to be redistributed through growing cells as the need arises, shifting to sites of localised growth. Jiang *et al.* (1997) studied the redistribution of cofilin/ADF throughout root hairs as they extended, the enzyme concentrating at the growing tip of the hair. This localised redistribution of the cofilin/ADF enzyme lead to the hypothesis in this study that an increased localised concentration of cofilin/ADF would precede branching.

Cofilin/ADF was at first believed to act by severing F-actin filaments (Hayden *et al.*, 1993), however, more recently it has been suggested that it acts by increasing the cycling of actin microfilaments, increasing the association of G-actin at the barbed ends of microfilaments while simultaneously increasing the disassociation at the pointed ends (Rosenblatt *et al.*, 1997). Chen *et al.* (2003) found that NtADF1 activity in tobacco pollen tubes was regulated by phosphorylation of the enzyme, that the both the active, nonphosphorylated ADF and the inactive, phosphorylated state of the enzyme were maintained in the growing cells, regulated by the NtRac1 gene. Cofilin/ADF has been

shown to prefer slightly alkaline conditions. In the apical region of pollen tubes a localised pH gradient exists, cofilin/ADF activity is strongest in the alkaline band of this gradient, thus this is where most F-actin cycling occurs (Chen *et al.*, 2002).

The possible role of cofilin/ADF in hyphal branching, intended as part of this thesis, was abandoned due to time constraints. It was replaced with the simpler LatB trials, but the future conduction of this experiment would be useful in testing the validity of the results of this thesis.

Other Organisms

Another direction of possible future research would simply entail the repetition of these experiments with different organisms. The use of hyphal mats, rather than spores, for the experimental hyphae would confirm the results as universal for *Achlya bisexualis* growth. Hyphal mats are easier to both establish and mount, their larger mass giving them an increased resistance to those factors (heat, desiccation, mechanical damage) that proved fatal to the germinated spores. This ease of setting up the experimental slides would greatly decrease the turnaround time of induction trials, allowing more images to be obtained in a single day. Finally, *A. bisexualis*, while ideal for micromanipulation, is not a true fungus, genetically belonging to the algae (Cavalier-Smith, 1986), despite its morphological similarity to the hyphal fungi. Repeating the experimental work with a “true” fungus would be necessary to determine how universal the results of this thesis are.

References

- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (Eds.) (2002).** *Molecular Biology of the Cell* (fourth edition). Garland Science, New York, USA.
- Bartnicki-Garcia, S. and Lippman, E. (1972).** The bursting tendency of hyphal tips of fungi: Presumptive evidence for a delicate balance between wall synthesis and wall lysis in apical growth. *Journal of General Microbiology* **73**: 487-500.
- Brachewich, C.L. and Heath, I.B. (1998).** Radial F-actin arrays precede new hypha formation in *Saprolegnia* - implications for establishing polar growth and regulating tip morphogenesis. *Journal of Cell Science* **111**: 2005-2016.
- Callaham, D. A. and Hepler, P. K. (1991).** Measurement of free calcium in plant cells. In McCormack, J. G., Cobbold, P. H. (Eds) *Cellular Calcium: A Practical Approach*. IRL Press, Oxford, UK.
- Carlie, M. J. and Watkinson, S. C. (1994).** *The Fungi*. Academic Press Limited, London, UK.
- Chen C, Dickman M. B. (2002).** *Colletotrichum trifolii* TB3 kinase, a COT1 homolog, is light inducible and becomes localized in the nucleus during hyphal elongation. *Eukaryotic Cell* Aug 1:626-33.
- Chitcholtan, K. and Garrill, A. (2005).** A $\beta 4$ integrin-like protein co-localises with a phosphotyrosine containing protein in the oomycete *Achlya bisexualis*: Inhibition of tyrosine phosphorylation slows tip growth. *Fungal Genetics and Biology* **42**: 534-545
- Dean, S. (2004).** Does the cytoskeleton manipulate the auxin-induced changes in the structure and motility of the endoplasmic reticulum? MSc thesis, University of Canterbury.
- Engqvist-Goldstein, A. and Drubin, D. (2003).** Actin assembly and endocytosis: from yeast to mammals. *Annual Review of Cell and Developmental Biology*. **19**: 287-332
- Gow, N. A. R. and Gooday, G. W. (1987).** Effects of antheridiol on growth, branching and electrical currents of hyphae of *Achlya ambisexualis*. *Journal of General Microbiology* **133**, 3531-3535.
- Harris, S.D. and Momany, M. (2004).** Polarity in filamentous fungi: moving beyond the yeast paradigm. *Fungal Genetics and Biology* **41**: 391-400.

- Harold, R. L., Money, N. P. and Harold, F. M. (1996).** Growth and morphogenesis in *Saprolegnia ferax*: is turgor required? *Protoplasma* **191**: 105-114.
- Hayden, S. M., Miller, P. S., Brauweiler, A. and Bamburg, J. R. (1993).** Analysis of the interactions of actin depolymerising factor with G- and F-actin. *Biochemistry*. **32**: 9994-10004.
- Heath, I. B. (1987).** Preservation of a labile cortical array of actin filaments in growing hyphal tips of the fungus *Saprolegnia ferax*. *European Journal of Cell Biology* **44**: 10-26
- Heath, I. B. (1995).** Integration and regulation of hyphal tip growth. *Canadian Journal of Botany* (Suppl. 1): S131-S139.
- Heath, I. B., Gupta, G and Bai, S. (2000).** Plasma membrane-adjacent actin filaments, but not microtubules, are essential for both polarisation and hyphal tip morphogenesis in *Saprolegnia ferax* and *Neurospora crassa*. *Fungal Genetics and Biology* **30**, 45-62.
- Heath, I.B., Bonham, M., Akram, A. and Gupta, G.D. (2003).** The interrelationships of actin and hyphal tip growth in the ascomycete *Geotrichum candidum*. *Fungal Genetics and Biology* **38**(1): 85-97.
- Jackson, S.L. and Heath, I.B. (1993).** UV microirradiation implicates F-actin in reinforcing growing hyphal tips. *Protoplasma*. **175**: 67-74.
- Jackson, S.L., Morris, E.J. and Garrill, A. (2001).** Hyphal branching and the induction of cell polarity. In A. Geitmann, M. Cresti, I.B. Heath, (Ed.s); *Cell Biology of Plant and Hyphal Tip Growth*. (NATO science series). (pp.69-79) Netherlands: IOS Press.
- Kropf, D. L. (1997).** Induction of polarity in fucoid zygotes. *The Plant Cell* **9**: 1011-1020.
- Mullins, J.T. (1979).** A freeze-fracture study of hormone-induced branching in the fungus *Achlya*. *Tissue & Cell* **11**(3): 585-595.
- Morris, E. J. S. (1999).** The involvement of Calcium in hyphal initiation and outgrowth in *Achlya*. MSc. Thesis, University of Canterbury.
- Robson, G. D., Wiebe, M. G. and Trinci, A. P. J. (1991).** Involvement of Ca^{2+} in the regulation of hyphal extension and branching in *Fusarium graminearum* A 3/5. *Experimental Mycology* **15**: 263-272.
- Schatten, G., Schatten, H., Spector, I., Cline, C., Paweletz, N., Simerly, C. and Petzelt, C. (1986).** Latrunculin inhibits the microfilament-mediated processes during fertilization, cleavage and early development in sea urchins and mice. *Experimental cell research*. **166**: 191-208.

Schreurs, W. J. A., Harold, R. L. and Harold, F. M. (1989). Chemotropism and Branching as Alternative Responses of *Achlya bisexualis* to Amino Acids. *Journal of General Microbiology* **135**: 2519-2528.

Trinci, A. P. (1971). Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media. *Journal of General Microbiology* **67**: 325–344.

Trinci, A. P. J., Weibe, M. G. and Robson, G. D. (1994). The mycelium as an integrated entity. In Wessels, J. G. H., Meinhardt, F. (Eds.) *The Mycota I: Growth, Differentiation and Sexuality*. Springer-Verlag, Berlin, Germany.

Utsugi, T., Minemura, M., Hirata, A., Abe, M., Watanabe, D. and Ohya, Y. (2002). Movement of yeast 1,3-beta-glucan synthase is essential for uniform cell wall synthesis. *Genes Cells*. **7**, 1-9.

Varvarigos, V., Katsaros, C. and Galatis, B. (2004). Radial F-actin configurations are involved in polarization during protoplast germination and thallus branching of *Macrocystis pyrifera* (Phaeophyceae, Laminariales) *Phycologia*. **Vol.43**(6): 693-703.

Walker, S. K. (2004). Changes to the cytoskeleton and cell wall underlie invasive hyphal growth. MSc thesis, University of Canterbury.

Wessels, J. G. H. (1986). Cell wall synthesis in apical hyphal growth. *International Review of Cytology* **104**: 37-79.

Young, M., Cooper, J. and Bridgman, P. (2004). Yeast actin patches are networks of branched actin filaments. *Journal of Cell Biology*. **166**: 629-635.

Yu, P.Y., Jackson, S.L. and Garrill, A. (2004). Two distinct distributions of F-actin are present in the hyphal apex of the oomycete *Achlya bisexualis*. *Plant Cell Physiology*. **45**(3): 275-280.

Appendix 1

Recipes

Branch Induction Solution

DMA Broth (see below)

1.0mM Phenylalanine (total concentration)

2.5 μ M Latrunculin B (for inhibitor trials, final concentration used)

DMA

0.3% Glucose

1.43% Yeast Nitrogen Base

Ingredients:

0.60mM Calcium Chloride

1.79mM Magnesium Sulfate

2.09 μ M Manganese Sulfate (Monohydrate)

0.65 μ M Ferrous Chloride (Heptahydrate)

0.14 μ M Cupric Sulfate

0.73 μ M Sodium Molybdate (Dihydrate)

1.22 μ M Zinc Sulfate (Monohydrate)

7.12 μ M Boric Acid

0.53 μ M Potassium Iodide

6.47 μ M Potassium Phosphate

Amino Acid Solution

1.36mM Glutamic acid

0.20mM Isoleucine

0.20mM Threonine

0.20mM Valine

0.20mM Lysine

0.10mM Glycine

0.10mM Arginine

0.10mM Tyrosine

0.10mM Serine

0.05mM Histidine

0.02mM Tryptophan

0.22mM Methionine

0.20mM Phenylalanine

Fixative Solution

0.5% Methylgloxal
4% Formaldehyde
50mM PIPES buffer
5mM EGTA

PYG

0.3% Glucose
0.125% Peptone
0.125% Yeast Extract

